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The Biocide Triclosan Selects *Stenotrophomonas maltophilia* Mutants That Overproduce the SmeDEF Multidrug Efflux Pump

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The possibility that triclosan selects *Stenotrophomonas maltophilia* mutants overexpressing the multidrug resistance pump SmeDEF is analyzed. Five out of 12 triclosan-selected mutants were less susceptible to antibiotics than the wild-type strain and overproduced SmeDEF. Results are discussed in relation to current debates on the potential selection of antibiotic-resistant bacteria by household biocides.

Triclosan (Irgasan) is a broad-spectrum antimicrobial compound widely used in toothpastes, cleaning solutions, plastics, house fabrics, and coatings for hospital devices. Triclosan resistance can be due to mutations in genes encoding enoyl reductases, to changes in membrane permeability, and/or to the expression of efflux pumps (13). The fact that some efflux pumps capable of extruding triclosan are capable also of extruding antibiotics (7, 8, 12) has produced some concern in the scientific community (10). If triclosan can select mutants that overproduce multidrug resistance (MDR) pumps, one of the highest risks for the emergence of antibiotic-resistant populations will be bacteria with an environmental origin, since one of the widest utilization of triclosan is in household products. In the last years we have characterized the SmeDEF efflux pump from *Stenotrophomonas maltophilia* (2), an opportunistic bacterial pathogen with an environmental origin. It has been shown that SmeDEF has a relevant role in both intrinsic (15) and acquired antibiotic resistance (3) in *S. maltophilia*. Herein we have analyzed whether triclosan might select *S. maltophilia* mutants that overproduce SmeDEF. To that goal, 100 μl of overnight cultures of *S. maltophilia* strain D457, grown in Luria-Bertani (LB) broth (6), was poured onto Mueller-Hinton plates (6) containing triclosan (64 μg/ml). From these plates, 12 mutants resistant to the biocide were picked up and grown in LB agar plates without antibiotics to avoid any possible induction of SmeDEF, and their susceptibility to tetracycline was tested by disk plate assays. Five of the mutants had reduced tetracycline susceptibility in comparison with the parental strain. The susceptibilities to different antibiotics of these five mutants were determined, and the results are shown in Table 1. All mutants were less susceptible to tetracycline, chloramphenicol, and ciprofloxacin, whereas the tobramycin MIC was lower or did not change (strain EM5). This phenotype is similar to that of the SmeDEF-overproducing strain D457R (4) and is thus compatible with SmeDEF overproduction in these mutants. To test this possibility, the expression of *smeD* (the first gene of the operon) was evaluated by reverse transcriptase PCR (RT-PCR). Briefly, 100 ng of total RNA from *S. maltophilia* grown to an optical density of 0.3 (λ = 600 nm) was subjected to RT-PCR analysis using the Ready-To-GO RT-PCR bead kit (Amersham Biosciences) by following the manufacturer's instructions. Primers smeD1 (5′-CCA AGAGCCTTTCCGTCAT-3′) and smeD2 (5′-TCTCGGACT TCAGCGTGAC-3′) were used (3) to test SmeDEF efflux pump expression in these mutants. To ascertain that no residual DNA was present in the RNA preparations, PCRs were performed under the same conditions except that no RT was added. The RT-PCR products were visualized in 2% agarose-ethidium bromide gels. As shown in Fig. 1a, all antibiotic-resistant triclosan mutants expressed *smeD* at higher levels than the wild-type parental strain. To further confirm these data, the level of expression of SmeF, the porin of the system, was analyzed by Western blotting as described previously (2). Whole-cell extracts from *S. maltophilia* strains, obtained from stationary-phase cultures and containing equal amounts of proteins, as measured with bicinchoninic acid systems (Pierce), were subjected to polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane (Millipore), stained with Ponceau S to confirm that equal amounts of protein had been loaded in each track, and analyzed with a polyclonal antibody raised against SmeF at a final dilution of 1:5,000. Horseradish peroxidase-conjugated protein A (Sigma) was used at a final concentration of 0.25 μg/ml, and detection of immunoreactive bands was performed by chemiluminescence with the commercial kit ECL-plus (Amersham Biosciences) according to the manufacturer's instructions. The results are shown in Fig. 1b and indicate that the triclosan-selected mutants overexpressed SmeF. Altogether these data indicate that triclosan can select (at least in vitro) *S. maltophilia* mutants that overproduce the multidrug efflux pump SmeDEF.

Selection of antibiotic-resistant mutants by widely used biocides has produced a strong debate in the last years (1, 11, 13). Several articles have indicated the risks of using antibacterial household products without any restriction. In fact, work carried out in vitro has demonstrated that biocides are able to select bacteria overexpressing multidrug efflux pumps (i.e., AcrAB [12] in *Escherichia coli* and MexCD-OprJ [7] and MexJK [8] in *Pseudomonas aeruginosa*). Our results are in line

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TABLE 1. Antibiotic susceptibility of *S. maltophilia* triclosan-resistant mutants

Strain	MIC (μg/ml)[a] of:				
	TET	CHL	CIP	TOB	TRI
D457[b]	8	16	4	32	64
D457R[b]	16	64	32	8	>256
EM1	16	32	32	4	>256
EM2	32	32	64	8	>256
EM3	32	32	32	16	>256
EM4	32	32	64	16	>256
EM5	32	32	64	32	>256

[a] Abbreviations: TET, tetracycline; CLOR, chloramphenicol; CIP, ciprofloxacin; TOB, tobramycin; TRI, triclosan.
[b] Control strain.

with those previous reports. There are two groups of *S. maltophilia* triclosan-resistant mutants. One group is formed by mutants in which the antibiotic susceptibility was unaffected, and the other is formed by mutants in which the antibiotic susceptibility was reduced as the consequence of SmeDEF overexpression. A recent work has shown that there is not a correlation between in-house utilization of common antibacterial cleaning agents and the presence of antibiotic-resistant bacteria in the home environment (9). Two hypothesis may explain the discrepancies between in vitro and in vivo data. First, the probability of emergence and enrichment of resistant populations in vitro might be different from that in vivo. Second, multidrug-resistant mutants may be impaired for survival in the environment (5, 14). In this case, even if these mutants are selected, they should be displaced by other triclosan-resistant strains that are not resistant to antibiotics. In vitro studies, like the one presented here, are useful for predicting the capability of an organism to become resistant in the future. The lack of correlation between antibiotic resistance and the utilization of housecleaning antibacterials in published field studies (9) reflects the current situation. However, as stated in reference 13, "There remain concerns about the unnecessary use of triclosan and other biocides in the home and in clinical settings." More studies are required to understand the behavior in natural environments of antibiotic-resistant mutants selected by triclosan in order to predict the future trend of the association between triclosan and antibiotic resistance.

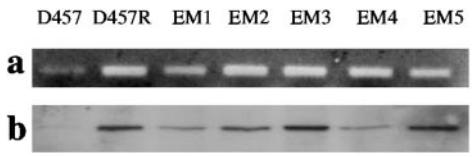

FIG. 1. Expression of SmeDEF by triclosan-selected *S. maltophilia* mutants. The level of expression of SmeDEF by triclosan-selected mutants was analyzed by RT-PCR and Western blotting. The strains D457 (wild type) and D457R (SmeDEF overproducer) were included as controls in the analysis. (a) Analysis of *smeD* expression in triclosan-resistant strains. The expression of *smeD* was estimated by RT-PCR. All triclosan-resistant mutants expressed higher levels of *smeD* than wild-type strain D457 and levels similar to that produced by SmeDEF-overproducing strain D457R. (b) Analysis of SmeF expression in triclosan-resistant strains. The expression of SmeF was estimated by Western blotting with an anti-SmeF antibody. All triclosan-resistant mutants expressed higher levels of SmeF than wild-type strain D457 and levels similar to that produced by SmeDEF-overproducing strain D457R.

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